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(54) Title: REAGENT AND METHOD FOR SERUM IRON ASSAY

## (57) Abstract

An automatable method utilizing a novel reagent for the assay (determination) of serum iron. The serum iron assay reagent is a single, stable reagent, which includes the chromogen chromazurol B (CAB), chromazurol S (CAS), or an alkali metal, alkaline earth metal, rubidium or ammonium base addition salt of CAB or CAS, a surfactant that forms a ternary complex with iron and the chromogen, a buffer, and dimethylsulfoxide.

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REAGENT AND METHOD FOR SERUM IRON ASSAY

This invention relates to the assay of serum iron in blood serum or heparinized plasma.

In the blood, iron in its ferric oxidation state is bound to the protein transferrin. Normally, transferrin is only partially saturated with iron and serves to transport iron from the digestive tract to the blood forming organs, where iron is removed from transferrin and incorporated into hemoglobin. Unsaturated iron binding capacity (UIBC) is the measurement of the amount of transferrin that carries no iron, while serum iron is the measurement of the amount of iron that is bound to transferrin.

The assays of serum iron and UIBC are useful in diagnosing, differentiating and predicting various forms of anemia. The measurement of serum iron in combination with the measurement of UIBC allows the physician to determine which part of the body's pathway to hemoglobin synthesis is deficient, and with such knowledge the treatment of the anemia becomes apparent.

Abnormal serum iron and UIBC levels can be used to predict that anemia will occur in certain pregnancies due to poor absorption of folic acid from the digestive tract. The reason that these serum iron/UIBC changes occur before anemia has developed is due to the long half-life of erythrocytes in the blood. The hemoglobin level represents the adequacy of hemoglobin formation over several weeks prior to hemoglobin measurement, and serum iron/UIBC measurements indicate the current status of hemoglobin formation. Therefore, serum iron/UIBC measurements allow therapy to be implemented before anemia develops, thereby preventing injury to the mother or fetus.

Traditionally, serum iron has been assayed by adding a serum sample to a reagent buffered at an acid pH, which dissociated ferric ion from transferrin. The reagent included a reducing agent, which aided in the dissociation process and reduced ferric ion to ferrous ion. A chromo-

genic reagent was then added and the chromogen complexed with ferrous iron to form a colored complex, which was measured spectrophotometrically.

However, traditional serum iron assays have been problematic for several reasons. First, serum contains chromatic components, such as hemoglobin and bilirubin, which significantly absorb light at the measuring absorption wavelength of the iron-chromogen colored complex. Second, turbidity due to lipemia interferes with spectral measurements of serum iron. Third, reducing agents, such as thioglycollic acid and ascorbic acid, used in the assay are unstable. Fourth, cupric ion found in serum can compete with ferrous ion for complexing with the chromogen. Fifth, some serum components, such as bilirubin, citric acid, oxalic acid, phospholipids and proteins, can bind serum iron, thus preventing serum iron from complexing with the chromogen.

Certain improvements were made to the serum iron assay by Garcic, Clin. Chim. Acta, 94, 115-19 (1979) (hereinafter Garcic). Garcic discloses the use of the ammonium salt of the chromogen chromazurol B (also known as chrome azurol B; eriochrome azurol B; and mordant blue 1) (hereinafter CAB), and the surfactant cetyltrimethylammonium bromide (CTMA), which react with ferric ion to form a colored complex. The CAB-CTMA-iron ternary complex is spectrophotometrically measured at 630 nanometers (nm), a wavelength at which the chromatic components of serum either no longer interferingly absorb light or minimally absorb light (in the case of turbid samples). Further, Garcic claims cupric ion interference is negligible, which is disputed by Torelli (U.S. Patent No. 4,810,656, col. 1, lines 11-26), and reducing agents are avoided. However, the serum iron assay requires a 20 minute reaction time, which is too long for automation. Although some automated analyzers can perform an assay that requires a reaction time longer than about 10 minutes, the overwhelming majority of commercially available automated analyzers

(such as the Hitachi 704, 707, and 747 analyzers, and the Olympus AU 5000) cannot perform an assay that requires a reaction time longer than about 10 minutes, and a reaction time of less than 10 minutes is greatly preferred (see 5 Takano et al., U.S. Patent No. 4,588,695, col. 3, lines 29-31). Automation of the serum iron assay is highly desirable because it is one of the twenty most commonly performed medical diagnostic tests in clinical laboratories.

10 Another problem with the Garcic method is that protein interference is substantial. Protein interference must be subtracted from the measurement of a test sample by employing a serum blank comprised of serum, chromogenic reagent and a masking reagent that includes citric acid, 15 which prevents formation of the CAB-CTMA-iron complex.

The Garcic method requires the preparation of the ammonium salt of CAB. Since the acid form of CAB is poorly soluble in water, the ammonium salt of CAB is prepared to impart sufficient solubility to CAB in aqueous 20 solutions. To avoid the necessity of preparing the ammonium salt, Tabacco et al., U.S. Patent No. 4,407,962 ('962), improved the Garcic method by adding ethanol to the chromogenic reagent to solubilize CAB in its acid form. Therefore, preparation of the ammonium salt of CAB 25 is no longer necessary. However, a 20-minute reaction time is still required for the assay. Further, a serum blank with masking reagent is required to compensate for protein interference.

Tabacco et al., U.S. Patent No. 4,703,015 ('015), 30 discloses use of the chromogen chromazurol S (also known as chrome azurol S; mordant blue 29; and chromeazurol S) (hereinafter CAS) as a substitute for CAB in the chromogenic reagent for a serum iron assay. Although CAS has a slightly lower molar coefficient of extinction (molar 35 absorptivity) than CAB, CAS is more soluble in aqueous medium and the addition of an organic solvent to the chromogenic reagent is not required. However, assay

reaction time is 20 minutes; therefore, the assay is not automatable. Further, protein interference remains a problem, thus requiring a serum blank that includes a masking reagent, and interference from cupric ion may 5 still be significant. Torelli, col. 1, lines 11-26.

Torelli discloses a chromogenic reagent for serum iron assay that eliminates protein and cupric interferences. The reagent includes CAB, a surface active agent (such as CTMA) in a concentration of at least 500 10 milligrams (mg)/liter (l), a salt (preferably sodium chloride) in a concentration that imparts an ionic strength of at least 100 grams (g)/l (expressed in terms of sodium chloride concentration), and an amino acid, such as glycine, to eliminate cupric ion interference. However, the high salt concentration of this reagent is 15 disadvantageous for automation because many automated analyzers wash and reuse reaction vessels. If sodium chloride is used, any salt residue remaining after washing of the reaction vessel will artificially elevate a subsequent serum analysis for sodium and chloride. Further, 20 the high concentration of surface active agent lowers the molar absorptivity of the CAB-CTMA-iron ternary complex.

Denney et al., U.S. Patent No. 4,224,034 ('034), and Outcalt et al., U.S. Patent No. 4,154,929 ('929) disclose 25 the use of dimethylsulfoxide (DMSO) in a serum iron assay to speed up the assay and to diminish turbidity due to lipemia and/or protein precipitation. '034 col. 4, lines 58-61 and col. 8, lines 33-42; '929 col. 4, lines 58-61 and col. 8, lines 38-47. The '034 and '929 patents 30 do not teach the addition of DMSO to eliminate nonturbidity related protein interferences because a serum blank measurement is required by the assay methods. '034 col. 8, lines 43-61; '929 col. 8, lines 48-66. Further, the serum iron assay methods taught by the '034 and '929 35 patents employ the unstable reducing agent ascorbic acid. Also, the percentage of DMSO employed in the serum iron assay taught by '034 and '929 is 7% (vol.:vol.) in the

final reaction mixture (10% vol.:vol. in the acetate buffer used in the assay). '034 col. 8, lines 45-50; '929 col. 8, lines 50-55.

One object of the invention is to provide a relatively quick serum iron assay that may be automated.

Another object of the invention is to provide a single, stable reagent for use in a serum iron assay.

Another object of the invention is to provide a serum iron assay that does not require a serum blank for the subtraction of protein interference.

Another object of the invention is to provide a serum iron assay that avoids interference from cupric iron.

Another object of the invention is to avoid incorporating unstable reducing agents into a reagent for serum iron assay.

Still other objects and advantages of the invention will become apparent to those of skill in the art after reading the following description of a preferred embodiment.

The invention provides an automatable method utilizing a novel reagent for the assay (determination) of serum iron.

The serum iron assay reagent is a single, stable reagent, which includes the chromogen chromazurol B (CAB), chromazurol S (CAS), or an alkali metal, alkaline earth metal, rubidium, or ammonium base addition salt of CAB or CAS, a surfactant that forms a ternary complex with iron and the chromogen, a buffer and dimethylsulfoxide. The inclusion of dimethylsulfoxide provides the multiple advantages of solubilizing the chromogen in its acid-form, eliminating interference from protein in serum, thereby eliminating the need for a serum blank with a masking reagent, and accelerating assay time, thereby making the serum iron assay automatable.

I. Serum Iron Reagent

The serum iron reagent is a single, stable reagent, meeting the following general requirements:

General Requirements of Stock Serum Iron Reagent

	<u>Amount of Ingredient per liter (l) of Aqueous Reagent</u>
5 <u>Ingredient</u>	
10    chromogen (CAB, CAS, or alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof)	From about 0.01 to about 0.3 millimol (m mol)
15    a complexing surfactant (an alkylammonium halide or polyhydroxyalkylene ether)	about a 1:1 molar ratio of complexing surfactant to chromogen is preferred
20    a buffer that will not complex with iron	sufficient to maintain a pH of from about 4.5 to about 5.5 when the reagent is added to a serum sample
25    dimethylsulfoxide (DMSO)	from about 100 ml to about 300 ml
30	The chromogen must be provided in sufficient concen- tration to react with all iron in a serum sample. CAB, CAS and their alkali metal, alkaline earth metal, rubidium and ammonium salts are highly sensitive chromogens (CAB and CAS have a high molar absorptivity.). However, CAB and its salts are preferred because of their slightly greater sensitivity (greater molar absorptivity).
35	A complexing surfactant, either an alkylammonium halide or a polyhydroxyalkylene ether, is added to the reagent to form the ternary complex, chromogen-complexing surfactant-iron, which is spectrophotometrically measured in the assay. The complexing surfactant must be provided in sufficient concentration so that it will react with all the binary complex, chromogen-iron, in the serum sample. Optimally, the molar ratio of complexing surfactant to chromogen is about 1:1. Increasing the amount of complexing surfactant above the 1:1 ratio only decreases the molar absorptivity of the ternary complex. Decreasing the amount of complexing surfactant below the 1:1 ratio runs the risk of having insufficient surfactant to form all of the ternary complex that can be formed, based upon

the amount of iron available for complexation in a serum sample. The preferred complexing surfactant is cetyltrimethylammonium bromide (CTMA), also known as hexadecyltrimethylammonium bromide, which forms the ternary complex of 5 CAB-CTMA-iron. The chromogen-complexing surfactant-iron complex is measured in the assay at a wavelength ( $\lambda$ ) from about 630 nanometers (nm) to about 660 nm with a  $\lambda$  of about 640 nm being preferred.

The inclusion of DMSO provides several advantages for 10 the reagent and the assay of serum iron. DMSO shortens assay reaction time to a range of from about 3 minutes to about 10 minutes, thereby making the assay automatable on commercially available automated analyzers, which require assay reaction times of about 10 minutes or less.

15 DMSO solubilizes the acid-form of CAB. Therefore, preparation of an alkali metal, alkaline earth metal, or ammonium base addition salt of CAB is not required. However, if desired, a base addition salt of CAB may be used. Garcic (at page 116) discloses preparation of the 20 ammonium salt of CAB. The preparation of other base addition salts of CAB is also contemplated by treatment of CAB with alkali metal and alkaline earth metal bases. For example, CAB may be treated with sodium hydroxide or potassium hydroxide to form a base addition salt.

25 Further, DMSO eliminates interference in the assay from serum protein. Therefore, a serum blank with or without a masking reagent is not required.

Although the amount of DMSO per liter of reagent may range from about 100 ml to about 300 ml, the preferred 30 amount is about 200 ml. At about 200 ml per liter of reagent, the speed of the assay and protein interference are at a minimum. When the concentration of DMSO increases above about 300 ml/l reagent, the assay improves little, if any, and reagent cost increases due to the 35 expense of DMSO.

Buffers, such as dicarboxylic acids, tricarboxylic acids (e.g. citric acid), ethylenediaminetetraacetic acid,

and phosphates, that will complex with iron should be avoided. Any buffer that will provide a pH of from about 4.5 to about 5.5 and that will not complex with iron may be used. An acetic acid/sodium acetate buffer will 5 achieve this pH range, will not complex with iron, and is preferred because it is inexpensive.

Although interference from cupric ion has not been observed in the assay of serum iron based upon the above reagent, thiourea, which prevents interference from cupric 10 ion, may be optionally included in the serum iron reagent in at least about 0.1 g thiourea/l of serum iron reagent to safeguard against cupric ion interference in the serum sample.

Because serum components, such as bilirubin, citric 15 acid, oxalic acid, phospholipids, and proteins have the potential for binding serum iron, an inorganic alkali metal salt or an alkaline earth metal salt, preferably a magnesium halide and most preferably magnesium chloride, is added to the serum iron reagent to provide these serum 20 components with a cationic complexing agent other than iron. The addition of such a salt to the reagent is preferred but not required.

A preferred serum iron reagent is prepared by the following steps:

25 1) add about 765 mg of CAS (available from Chemical Dynamics and from Fluka Chemika-BioChemika; molecular weight = 605.29) to about 200 ml of DMSO and mix to form a first solution;

30 2) add iron-free water to the first solution to bring the total volume to about 800 ml, thereby forming a second solution;

35 3) add from about 0.33 mol to about 0.39 mol potassium hydroxide or sodium hydroxide and about 37 g of glacial acetic acid to the second solution and mix until dissolved, thereby forming a third solution;

- 4) add about 270 mg of CTMA to the third solution and stir until dissolved, thereby forming a fourth solution;
- 5) add about 20 g of magnesium chloride hexahydrate (about 98 m mol magnesium chloride) to the fourth solution and stir until dissolved, thereby forming a fifth solution;
- 6) add iron-free water to the fifth solution until a volume of 1 liter is achieved, thereby forming the serum iron reagent.

10 The inventive reagent may also be supplied as a diagnostic kit for use in automated chemical analyzers, particularly the Hitachi 705 automated biochemistry analyzer.

15 Generally, the diagnostic kit is comprised of first and second compositions. At a minimum, the first composition includes a first portion of DMSO and iron-free water. The second composition includes a second portion of DMSO, chromogen, buffer, complexing surfactant, and iron-free water. When the first and second compositions are combined, as described in Serum Iron Assay Method below, the concentration of DMSO is within the above-stated concentration range for the inventive reagent (from about 100 ml DMSO to about 300 ml DMSO per liter of reagent).

20 25 More preferably, the first composition will include thiourea to safeguard against cupric ion interference, and a polyoxyethylenesorbitan surfactant. The second composition will more preferably include thiourea and an inorganic salt selected from the group described above.

30 35 Most preferably, the polyoxyethylenesorbitan surfactant will be polyoxyethylenesorbitan monooleate (TWEEN 80 available from Sigma Chemical Company).

A particularly preferred diagnostic kit for the Hitachi 705 analyzer may be made as follows:

35 First Composition (R1)

Step 1 - Add iron-free water to about 192 g DMSO until a volume of about 500 ml is achieved.

Step 2 - Add about 0.1 g thiourea and about 0.5 ml polyoxyethylenesorbitan monooleate and mix until a solution is formed.

Step 3 - Add iron-free water until a volume of about 5 one liter is achieved.

Second Composition (R2)

Step 1 - Mix about 436 g DMSO with about 1 m mol CAB (about 635 mg of approximately 80% CAB available from Sigma Chemical Company) until a solution is formed.

10 Step 2 - Add iron-free water until a total volume of about 750 ml is achieved.

Step 3 - Add from about 1.3 mol to about 1.6 mol of potassium hydroxide or sodium hydroxide, about 147 g glacial acetic acid, about 0.1 g thiourea, about 2 g 15 hexadecyltrimethylammonium bromide, about 2 g magnesium chloride and mix until a solution is formed.

Step 4 - Add iron-free water until total volume is about one liter.

Substituting an equivalent molar amount of any ingredient above with another ingredient within the same category given under General Requirements of Stock Serum Iron Reagent (i.e., any of the chromagens, complexing surfactants, and buffers listed under General Requirements of Stock Serum Iron Reagent) will yield an acceptable diagnostic kit that may be used in an automatable assay.

II. Serum Iron Assay Method

An assay for serum iron requires the serum iron reagent (disclosed above) and an iron standard containing a known amount of iron. At a minimum, the iron standard 30 for an iron assay that utilizes the chromogens CAS and CAB (and their salts as described above) should contain transferrin, ferric ion from a ferric salt, such as ferric chloride, and iron-free water.

A preferred iron standard may be prepared by adding 35 iron-free water to a known amount of ferric ions (for example, from ferric chloride) and an additive, such as hydrochloric acid, that helps prevent reduction of ferric

ion to ferrous ion, to form an iron-containing solution of known volume. The ferric ions must be in sufficient amount to achieve a concentration of ferric ions within the dynamic concentration range for iron in serum (the 5 concentration range of iron that will be found in serum). A satisfactory iron-containing solution contains 400 micrograms ( $\mu\text{g}$ ) of iron per deciliter (dl) of solution. Such a solution may be prepared by adding 116.2 mg ferric chloride (anhydrous) to a 10 l volumetric flask.

10 Ten (10) ml of concentrated hydrochloric acid is then added to the flask followed by the addition of deionized water until a volume of 10 l is obtained.

Generally, the assay method is comprised of the following steps:

- 15     a. adding a serum sample to the serum iron reagent followed by mixing to form a test sample;
- b. adding iron-free water to the serum iron reagent followed by mixing to form a test blank;
- c. adding iron standard to the serum iron reagent 20 to form a test standard;
- d. incubating the test sample, the test blank, and the test standard at about 37°C from about 3 minutes to about 10 minutes;
- e. spectrophotometrically determining absorbances 25 (A) of the test sample, the test blank, and the test standard; and
- f. calculating the concentration of iron in the test sample by the following equation:

$$\frac{A_{\text{test sample}} - A_{\text{test blank}}}{A_{\text{test standard}} - A_{\text{test blank}}} \times \frac{\text{known concentration}}{\text{of iron in the test standard}} = \text{concentration of iron in the test sample.}$$

- 35 Absorbances are preferably measured at 640 nanometers (nm), and may be measured on automated chemistry analyzers using filters having mean wavelength band passes from about 630 nm to about 660 nm.

Importantly, the assay requires only about a three minute incubation period (reaction time), thereby easily making the method automatable. Further, the assay does not require the use of a serum blank with or without a 5 masking reagent for the subtraction of protein interference. The serum iron reagent employed in the assay eliminates such protein interference, thereby making the assay method simpler and more accurate than other serum iron assay methods.

10 Lipemia in serum can still cause error in the assay due to turbidity. Such error can be corrected by automated bichromatic correction means involving spectrophotometrically determining absorbances of the test sample and test blank at a primary wavelength ( $1^\circ\lambda$ ) from about 15 630 nm to about 660 nm (preferably at about 640 nm), followed by spectrophotometric measurement of those samples at a secondary wavelength ( $2^\circ\lambda$ ) from about 680 nm to about 850 nm (preferably at about 700 nm). Correction for turbidity in lipemic serum is then made by the 20 following calculation which is substituted for the absorbance measurements in the numerator of the above equation for determining concentration of the test sample:

$$\frac{(A \text{ test sample } 1^\circ\lambda - A \text{ test blank } 1^\circ\lambda)}{(A \text{ test sample } 2^\circ\lambda - A \text{ test blank } 2^\circ\lambda)}$$

25 This bichromatic measurement corrects for error due to turbidity because absorbance due to turbidity remains relatively constant from the  $1^\circ\lambda$  to the  $2^\circ\lambda$ , while absorbance of the chromogen-complexing surfactant-iron complex decreases dramatically from the  $1^\circ\lambda$  to the  $2^\circ\lambda$ . Therefore, subtraction of absorbances at  $2^\circ\lambda$  from absorbances 30 at  $1^\circ\lambda$  yields absorbance due to the chromogen-iron-surfactant complex.

Another means of correcting lipemic serum samples is to analyze a separate serum blank without a masking reagent. The serum blank without masking reagent would 35 include a second serum sample (same volume and serum pool as serum sample in the test sample) and iron-free water (same volume as serum iron reagent) and would be incubated

and spectrophotometrically measured analogously to the test sample, test blank and test standard. The concentration of iron in the test sample would then be calculated by the following equation:

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$$A_{\text{test sample}} = (A_{\text{test blank}} + A_{\text{serum blank without masking reagent}})$$


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$$X \frac{A_{\text{test standard}} - A_{\text{test blank}}}{\text{known concentration of iron in the test standard}} = \frac{\text{concentration of iron in test sample}}{\text{concentration of iron in standard}}$$

15

Alternatively, incubation may be conducted at ambient temperature. However, the assay is faster and more accurate at 37°C (see Tabacco et al., Clin. Chim. Acta, 114, 287, 289 (Table II) (1981)). Heparinized plasma may also be used instead of serum for the test sample and the serum blank without masking reagent.

20

Specifically, an assay may be performed with the preferred serum iron reagent by employing the following amounts of ingredients in the method stated above: 2 ml serum iron reagent, 0.1 ml iron-free water, 0.1 ml iron standard, 0.1 ml serum sample.

25

A widely used clinical analyzer is the Hitachi 705 automated biochemistry analyzer. A serum iron assay method, which utilizes the present inventive reagent, may be performed using the Hitachi 705 analyzer. Use of the Hitachi 705 to perform an assay illustrates the use of both bichromatic correction means and dynamic serum blank means for correcting error due to lipemia. (In a dynamic serum blank, the same serum sample is sequentially used for both the spectrophotometric blank measurement and color measurement. In a true serum blank, as described above, the serum sample is divided into two portions, one portion is used for spectrophotometric blank measurement and the other portion is used for color measurement.)

35

To perform an assay on the Hitachi 705 analyzer, a serum sample is diluted with reagent R1 (see Serum Iron Reagent section) to form a test blank. Spectrophotometric readings are then performed on the blank at

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660 nm ( $1^\circ\lambda$ ) and 700 nm ( $2^\circ\lambda$ ). Following these spectro-photometric measurements, reagent R2 is added to form a test sample. The test sample includes the inventive reagent and is incubated for about 5 minutes. Following 5 incubation, spectrophotometric measurements are performed on the test sample at 660 nm ( $1^\circ\lambda$ ) and 700 nm ( $2^\circ\lambda$ ). The specific instrument settings for the Hitachi 705 analyzer are as follows:

Chemistry Parameters for the Hitachi 705

10	Test	:Iron
	Assay Code	:Endpoint
	Sample Volume ( $\mu\text{l}$ )	:20
	R1 Volume ( $\mu\text{l}$ )	:350
15	R2 Volume ( $\mu\text{l}$ )	:100
	R3 Volume	:
	Wavelength 1	:700 nm
	Wavelength 2	:660 nm
	Rgt.Blk.Abs.	:---
20	Rgt.Blk.Conc.	:0
	Std.Conc.	:***
	Factor	:---
	Std.Abs.Allowance	:10%
	Normal Range L	:
25	Normal Range H	:
	Abs.Limit (Rate)	:
	Control I.D. No.	:***

\*\*\*Denotes user specific settings

---Determined by instrument

Under the parameter settings given above, the analyzer performs bichromatic spectrophotometric measurements on the test blank, test sample, and a test standard of known iron concentration. The concentration of iron 35 in the test sample is calculated by the equation

$$\frac{(A_{\text{test sample } 1^\circ\lambda} - A_{\text{test blank } 1^\circ\lambda}) - (A_{\text{test sample } 2^\circ\lambda} - A_{\text{test blank } 2^\circ\lambda})}{A_{\text{test standard } 1^\circ\lambda} - A_{\text{test standard } 2^\circ\lambda}}$$

$$X \frac{\text{known concentration}}{\text{of iron in the test}} = \frac{\text{concentration}}{\text{of iron in}} \\ \text{standard} \qquad \qquad \qquad \text{test sample.}$$

CLAIMS

1. A reagent for the assay of iron in serum or heparinized plasma, comprising:
  - a. a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof;
  - b. a complexing surfactant selected from the group consisting of alkylammonium halides and polyhydrox-yalkylene ethers;
  - c. a buffer that will not complex iron and will provide a pH of from about 4.5 to about 5.5; and
  - d. dimethylsulfoxide in an amount sufficient to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma, and achieve an incubation time in the assay of from about 3 minutes to about 10 minutes.
2. The reagent of claim 1, wherein the amount of dimethylsulfoxide is from about 100 ml/l reagent to about 300 ml/l reagent.
3. The reagent of claim 2, wherein the complexing surfactant is cetyltrimethylammonium bromide.
4. The reagent of claim 3, wherein the buffer is sodium acetate or potassium acetate.
5. The reagent of claim 1, 2 or 3, further comprising an inorganic salt selected from the group consisting of alkali metal and alkaline earth metal salts.
6. The reagent of claim 5, wherein the amount of dimethylsulfoxide is from about 100 ml/l reagent to about 300 ml/l reagent.
7. The reagent of claim 6, wherein the inorganic salt is magnesium chloride.
8. The reagent of claim 5, further comprising at least about 0.1 g thiourea/l reagent.
- 35 9. The reagent of claim 1, wherein the chromogen component comprises about 765 mg chromazural S/l reagent; the dimethylsulfoxide is present in an amount of about

200 ml dimethylsulfoxide/l reagent; the buffer comprises from about 0.33 mol to about 0.39 mol potassium hydroxide or sodium hydroxide/l reagent and about 37 g glacial acetic acid/l reagent; the complexing surfactant component comprises about 270 mg cetyltrimethylammonium bromide/l reagent; and wherein the reagent further comprises about 98 m mol magnesium chloride/l reagent, and iron-free water in an amount sufficient to bring the reagent volume to about 1 liter.

10 10. The reagent of claim 9, further comprising at least about 0.1 g thiourea/l reagent.

11. An automatable method for the assay of iron in serum or heparinized plasma, comprising the steps of:

15 a. providing a reagent for the assay of said iron, wherein said reagent comprises: a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof; a complexing surfactant selected from the group consisting of alkyl-ammonium halides and polyhydroxyalkylene ethers; a buffer that will not complex iron and will provide a pH of from about 4.5 to about 5.5; and dimethylsulfoxide in an amount sufficient to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma, and achieve an incubation time in the assay of from about 20 3 minutes to about 10 minutes;

25 b. preparing a test sample by adding a serum sample or heparinized plasma sample to said reagent;

30 c. preparing a test blank by adding iron-free water to said reagent;

d. preparing a test standard by adding an iron standard of known iron concentration to said reagent;

35 e. incubating the test sample, the test blank, and the test standard at about 37°C for at least about three minutes;

f. spectrophotometrically measuring absorbances (A) of the test sample, the test blank, and the test standard; and

5 g. calculating the concentration of iron in  
the test sample by the equation

$$\frac{A_{\text{test sample}} - A_{\text{test blank}}}{A_{\text{test standard}} - A_{\text{test blank}}} \times \begin{matrix} \text{known concentration} \\ \text{of iron in the} \\ \text{test standard} \end{matrix}$$

10

concentration of iron in the test sample.

12. The automatable method of claim 11, wherein the amount of dimethylsulfoxide in said reagent is from about 15 100 ml/l reagent to about 300 ml/l reagent.

13. The automatable method of claim 12, wherein said reagent further includes an inorganic salt selected from the group consisting of alkali metal and alkaline earth metal salts.

20

14. The automatable method of claim 11, wherein said chromogen component comprises about 765 mg chromazural S/l reagent; said dimethylsulfoxide is present in an amount of about 200 ml dimethylsulfoxide/l reagent; said buffer comprises from about 0.33 mol to 25 about 0.39 mol potassium hydroxide or sodium hydroxide/l reagent and about 37 g glacial acetic acid/l reagent; said complexing surfactant component comprises about 270 mg cetyltrimethylammonium bromide/l reagent; and wherein said reagent further includes about 98 m mol magnesium chloride/l reagent, and iron-free water in an amount 30 sufficient to bring the reagent volume to about 1 liter.

15. The automatable method of claim 11, 12, 13 or 14, wherein said absorbances (A) are measured at a wavelength of from about 630 nm to about 660 nm.

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16. The automatable method of claim 11, 12, 13 or 14, wherein said absorbances (A) are measured at about 640 nm.

17. An automatable method for the assay of iron in lipemic serum or heparinized plasma, comprising the steps 40 of:

a. providing a reagent for the assay of said iron, said reagent comprising: a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof; a complexing surfactant selected from the group consisting of alkylammonium halides and polyhydroxalkylene ethers; a buffer that will not complex iron and will provide a pH of from about 4.5 to about 5.5; and dimethylsulfoxide in an amount sufficient to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma, and achieve an incubation time in the assay of from about 3 minutes to about 10 minutes;

b. preparing a test sample by adding a first serum sample or heparinized plasma sample to said reagent;

c. preparing a test blank by adding iron-free water to said reagent;

d. preparing a test standard by adding an iron standard of known iron concentration to said reagent;

e. preparing a serum blank or heparinized plasma blank without masking reagent by adding a second serum sample or heparinized plasma sample to iron-free water;

f. incubating the test sample, the test blank, the test standard, and the serum blank or heparinized plasma blank without masking reagent at about 37°C for at least about three minutes;

g. spectrophotometrically measuring absorbances (A) of the test sample, the test blank, the test standard, and the serum blank or heparinized plasma blank without masking reagent; and

h. calculating the concentration of iron in the test sample by the equation

$$A_{\text{test sample}} = (A_{\text{test blank}} + A_{\text{serum blank or plasma blank}})$$


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$$A_{\text{test standard}} - A_{\text{test blank}}$$

5

$$x \frac{\text{known concentration}}{\text{of iron in the test}} = \frac{\text{concentration of}}{\text{standard}} \text{iron in the test sample.}$$

10 18. The automatable method of claim 17, wherein the amount of dimethylsulfoxide in said reagent is from about 100 ml/l reagent to about 300 ml/l reagent.

15 19. The automatable method of claim 18, wherein said reagent further includes an inorganic salt selected from the group consisting of alkali metal and alkaline earth metal salts.

20 20. The automatable method of claim 17, wherein said chromogen component comprises about 765 mg chromazural S/l reagent; said dimethylsulfoxide is present in an amount of about 200 ml dimethylsulfoxide/l reagent; said buffer comprises from about 0.33 mol to about 0.39 mol potassium hydroxide or sodium hydroxide/l reagent and about 37 g glacial acetic acid/l reagent; said complexing surfactant component comprises about 270 mg cetyltrimethylammonium bromide/l reagent; and wherein said reagent further includes about 98 m mol magnesium chloride/l reagent, and iron-free water in an amount sufficient to bring the reagent volume to about 1 liter.

30 21. The method of claim 17, 18, 19 or 20, wherein said absorbances (A) are measured at a wavelength of from about 630 nm to about 660 nm.

22. The method of claim 17, 18, 19 or 20, wherein said absorbances (A) are measured at about 640 nm.

35 23. A diagnostic kit for the assay of iron in serum or heparinized plasma, comprising:

a. a first composition comprised of a first portion of dimethylsulfoxide and iron-free water; and

b. a second composition comprised of

(1) a chromogen selected from the group

40 consisting of chromazurol B, chromazurol S, and alkali

metal, alkaline earth metal, rubidium, or ammonium salts thereof;

(2) a second portion of dimethylsulfoxide, the first and second portions of dimethylsulfoxide being in a sufficient amount, when added together, to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma, and achieve an incubation time in the assay of about 5 minutes or less;

(3) a buffer that will not complex with iron and will provide a pH of from about 4.5 to about 5.5;

(4) a complexing surfactant selected from the group consisting of alkylammonium halides and polyhydroxyalkylene ethers; and

(5) iron-free water.

24. The diagnostic kit of claim 23, wherein the first composition further comprises at least about 0.1 g thiourea/l of first composition and about 0.5 ml of a polyoxyethylenesorbitan surfactant/l of first composition, and the second composition further comprises at least about 0.1 g thiourea/l of second composition and an inorganic salt selected from the group consisting of alkali metal and alkaline earth metal salts.

25. The diagnostic kit of claim 23, wherein said first composition per liter comprises about 192 g dimethylsulfoxide, about 0.1 g thiourea, about 0.5 ml polyoxyethylenesorbitan monooleate, and iron-free water; and wherein said second composition per liter comprises about 436 g dimethylsulfoxide, about 1 m mol of a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof, from about 1.3 mol to about 1.6 mol potassium hydroxide or sodium hydroxide, about 147 g glacial acetic acid, about 0.1 g thiourea, about 2 g hexadecyltrimethylammonium bromide, and about 2 g magnesium chloride.

26. The diagnostic kit of claim 25, wherein the chromogen is chromazurol B.

27. An automatable method for the assay of iron in serum or heparinized plasma, comprising the steps of:

5       a. preparing a test blank by adding a serum sample or heparinized plasma sample to a first composition comprised of a first portion of dimethylsulfoxide and iron-free water; and;

10      b. spectrophotometrically measuring absorbance of the test blank at a primary wavelength ( $1^{\circ}\lambda$ ) and a secondary wavelength ( $2^{\circ}\lambda$ );

15      c. preparing a test sample by adding the test blank to a second composition comprised of

15      (1) a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof;

20      (2) a second portion of dimethylsulfoxide, the first and second portions of dimethylsulfoxide being in a sufficient amount, when added together, to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma, and achieve an incubation time in the assay of about 5 minutes or less;

25      (3) a buffer that will not complex with iron and will provide a pH of from about 4.5 to about 5.5;

30      (4) a complexing surfactant selected from the group consisting of alkylammonium halides and polyhydroxyalkylene ethers; and

          (5) iron-free water.

35      d. spectrophotometrically measuring absorbance of the test sample at the primary wavelength ( $1^{\circ}\lambda$ ) and the secondary wavelength ( $2^{\circ}\lambda$ );

35      e. spectrophotometrically measuring absorbance of a test standard of known iron concentration at

the primary wavelength ( $1^{\circ}\lambda$ ) and the secondary wavelength ( $2^{\circ}\lambda$ );

f. calculating the concentration of iron in the test sample by the equation

$$5 \quad \frac{(A_{\text{test sample } 1^{\circ}\lambda} - A_{\text{test blank } 1^{\circ}\lambda}) - (A_{\text{test sample } 2^{\circ}\lambda} - A_{\text{test blank } 2^{\circ}\lambda})}{A_{\text{test standard } 1^{\circ}\lambda} - A_{\text{test standard } 2^{\circ}\lambda}}$$

$$10 \quad x \quad \frac{\text{known concentration of iron in the test}}{\text{concentration of iron in the standard}} = \frac{\text{concentration of iron in the test sample.}}$$

28. The method of claim 27, wherein said first composition per liter comprises about 192 g dimethylsulfoxide, about 0.1 g thiourea, about 0.5 ml polyoxyethylene sorbitan monooleate, and iron-free water; and wherein said second composition per liter comprises about 436 g dimethylsulfoxide, about 1 m mol of a chromogen selected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof, from about 1.3 mol to about 1.6 mol potassium hydroxide or sodium hydroxide, about 147 g glacial acetic acid, about 0.1 g thiourea, about 2 g hexadecyltrimethylammonium bromide, and about 25 2 g magnesium chloride.

29. The method of claim 27 or 28, wherein said primary wavelength is from about 630 nm to about 660 nm and said secondary wavelength is from about 680 nm to about 850 nm.

30. The method of claim 27 or 28, wherein said primary wavelength is about 640 nm and said secondary wavelength is about 700 nm.

31. An automatable method for the assay of iron in lipemic serum or heparinized plasma, comprising the steps of:

a. spectrophotometrically measuring the absorbance (A) of a dynamic blank of serum or heparinized plasma without masking reagent;

40 b. providing a reagent for the assay of said iron, wherein said reagent comprises: a chromogen se-

- lected from the group consisting of chromazurol B, chromazurol S, and alkali metal, alkaline earth metal, rubidium, or ammonium salts thereof; a complexing surfactant selected from the group consisting of alkyl-
- 5 ammonium halides and polyhydroxyalkylene ethers; a buffer that will not complex iron and will provide a pH of from about 4.5 to about 5.5; and dimethylsulfoxide in an amount sufficient to solubilize the chromogen, eliminate protein interference in serum or heparinized plasma,
- 10 and achieve an incubation time in the assay of from about 3 minutes to about 10 minutes;
- c. preparing a test blank by adding iron-free water to said reagent;
- d. preparing a test sample by adding the dynamic blank to said reagent;
- 15 e. preparing a test standard by adding an iron standard of known iron concentration to said reagent;
- f. incubating the test sample, test blank and test standard at about 37°C for at least about three
- 20 minutes;
- g. spectrophotometrically measuring absorbances (A) of the test sample, test blank and test standard; and
- h. calculating the concentration of iron in
- 25 the test sample by the equation

$$\frac{A_{\text{test sample}} - (A_{\text{test blank}} + A_{\text{dynamic blank}})}{A_{\text{test standard}} - A_{\text{test blank}}} \times$$

30 known concentration of iron in test standard = concentration of iron in the test sample

32. The automatable method of claim 31, wherein the amount of dimethylsulfoxide in said reagent is from about 35 100 ml/l reagent to about 300 ml/l reagent.

33. The automatable method of claim 31 or 32, wherein said reagent further includes an inorganic salt

selected from the group consisting of alkali metal and alkaline earth metal salts.

34. The automatable method of claim 31, wherein said chromogen component comprises about 765 mg chromazural S/l reagent; said dimethylsulfoxide is present in an amount of about 200 ml dimethylsulfoxide/l reagent; said buffer comprises from about 0.33 mol to about 0.39 mol potassium hydroxide or sodium hydroxide/l reagent and about 37 g glacial acetic acid/l reagent; said complexing surfactant component comprises about 270 mg cetyltrimethylammonium bromide/l reagent; and wherein said reagent further includes about 98 m mol magnesium chloride/l reagent, and iron-free water in an amount sufficient to bring the reagent volume to about 1 liter.

15 35. The method of claim 31, 32, 33 or 34, wherein said absorbances (A) are measured at a wavelength of from about 630 nm to about 660 nm.

36. The method of claim 31, 32, 33 or 34, wherein said absorbances (A) are measured at about 640 nm.

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**(54) Title:** REAGENT AND METHOD FOR SERUM IRON ASSAY

**(57) Abstract**

An automatable method utilizing a novel reagent for the assay (determination) of serum iron. The serum iron assay reagent is a single, stable reagent, which includes the chromogen chromazurol B (CAB), chromazurol S (CAS), or an alkali metal, alkaline earth metal, rubidium or ammonium base addition salt of CAB or CAS, a surfactant that forms a ternary complex with iron and the chromogen, a buffer, and dimethylsulfoxide.

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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 91/07477

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>4</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 G01N33/84; G01N33/90

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.C1. 5	G01N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	US,A,4 154 929 (M.C.OUTCALT ET AL.) 15 May 1979 cited in the application  See the abstract; Column 8, line 36- column 9 , line 16. ---	1-6, 11-13, 15-19, 21-23
Y	CLINICA CHIMICA ACTA vol. 94, 1979, NL pages 115 - 119; A.GARCIC: 'A highly sensitive, simple determination of serum iron using chromazurol b.' cited in the application see the whole document ---	1-6, 11-13, 15-19, 21-23

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## IV. CERTIFICATION

Date of the Actual Completion of the International Search

1 30 JULY 1992

Date of Mailing of this International Search Report

11.08.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

HITCHEN C.E.

C.Wd

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>CHEMICAL ABSTRACTS, vol. 101, no. 20, 12 November 1984, Columbus, Ohio, US; abstract no. 183012B, C.YUNXIANG ET AL.: 'Solvation effects of color reactions of multicomponent complexes-iron(III) - Chrome Azurol S - cetyltrimethylammonium bromide-oxygen-containing organic solvent systems.' page 760 ; column 2 ; see abstract &amp; Gaodeng Xuexiao Huaxue Xuebao 1984, 5(5), 635-640.</p> <p>---</p>	1-6, 11-13, 15-19, 21-23
A	<p>US,A,4 407 962 (A.TABACCO ET AL.) 4 October 1983 cited in the application see the whole document</p> <p>---</p>	1-7
A	<p>US,A,4 810 656 (G.TORELLI) 7 March 1989 cited in the application see the whole document</p> <p>---</p>	1-6

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. US 9107477  
SA 59893**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
 The members are as contained in the European Patent Office EDP file on  
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